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Rapid and Specific Detection of *Escherichia coli* Clonal Group A by Gene-Specific PCR

James R. Johnson,[1]* Krista Owens,[1]† Amee R. Manges,[2] and Lee W. Riley[2,3]

Mucosal and Vaccine Research Center, Minneapolis Veterans Affairs Medical Center, and Department of Medicine, University of Minnesota, Minneapolis, Minnesota,[1] and Division of Epidemiology, School of Public Health,[2] and Division of Infectious Diseases and Immunity,[3] University of California at Berkeley, Berkeley, California

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PCR primers specific for the recently described antimicrobial resistance-associated *Escherichia coli* clonal group A (CGA), a widespread cause of drug-resistant urinary tract infections in the United States, were devised on the basis of a novel single-nucleotide polymorphism identified within the housekeeping gene *fumC*, i.e., C288T. In comparison with two reference PCR-based fingerprinting methods, ERIC2 PCR and random amplified polymorphic DNA (RAPD) analysis, a PCR assay incorporating the new primers provided 100% sensitivity and 100% specificity for the detection of CGA among 138 diverse clinical and reference *E. coli* isolates. *E. coli* reference (ECOR) strain 47 was shown to be a member or a close relative of CGA (by ERIC2 PCR and RAPD analysis, respectively) and yielded a positive assay result. The new CGA-specific PCR assay, which exhibited interlaboratory reproducibility and stability under various experimental conditions, should allow the rapid and specific detection of CGA by any laboratory equipped for diagnostic PCR.

A single clonal group of extraintestinal pathogenic *Escherichia coli*, provisionally designated clonal group A (CGA), has recently been shown to account for 33 to 57% of episodes of acute uncomplicated cystitis and pyelonephritis due to trimethoprim-sulfamethoxazole-resistant *E. coli* among U.S. women (11, 15, 16). This newly emerged clonal group characteristically exhibits multidrug resistance and is broadly distributed across the United States, with some evidence of local point-source spread (2, 10, 11, 15, 16). In some locales, CGA alone contributes sufficiently to the prevalence of trimethoprim-sulfamethoxazole resistance to raise this value to above the 10 to 20% threshold at which alternative (e.g., fluoroquinolone-based) empirical therapy is recommended for acute cystitis (2, 16, 23). These observations demonstrate the current clinical importance of CGA and indicate a need for further molecular epidemiological analysis of the CGA phenomenon.

To date, CGA has been defined on the basis of the distinctive genomic banding patterns generated by PCR-based molecular fingerprinting methods, including repetitive element PCR, i.e., enterobacterial repeat intergenic consensus (ERIC) sequence PCR with the ERIC2 primer, and random amplified polymorphic DNA (RAPD) analysis with various arbitrary decamer primers (2, 10, 11, 15, 16). Unfortunately, PCR-based fingerprinting methods are highly condition dependent and, hence, suffer from run-to-run, preparation-to-preparation, cycler-to-cycler, and interlaboratory variabilities (5, 12, 21). They also require the subjective interpretation of banding patterns, which introduces an additional opportunity for error (3, 19, 22). Pulsed-field gel electrophoresis analysis provides better reproducibility but is too highly discriminating to reliably identify isolates as CGA because it tends to see different CGA isolates as clonally distinct, even though they are indistinguishable by PCR-based fingerprinting methods (11, 16). A more reproducible, objective, reliable, and portable diagnostic test for CGA would greatly facilitate the molecular epidemiological studies needed. Accordingly, we sought to develop a gene-specific PCR assay for the rapid and specific detection of CGA.

* Corresponding author. Mailing address: Infectious Diseases (111F), Minneapolis VA Medical Center, One Veterans Dr., Minneapolis, MN 55417. Phone: (612) 467-4185. Fax: (612) 727-5995. E-mail: johns007@umn.edu.

† Present address: Ecolab, St. Paul, Minn.

MATERIALS AND METHODS

Strains. Reference CGA isolates included UMN026 (a cystitis isolate from the University of Minnesota student health service) (16), SEQ102 (a cystitis isolate from the University of California at Berkeley; ATCC BAA-457) (16), and V10 and V11 (urosepsis isolates from Seattle, Wash.) (13). Comparison isolates for sequence analysis ($n = 28$) included representatives of several other recognized extraintestinal pathogenic *E. coli* clonal groups, including the closely related O15:K52:H1 clonal group (11, 14, 18) and selected members of the *E. coli* reference (ECOR) collection (17) representing all four major *E. coli* phylogenetic groups (groups A, B1, B2, and D) and ungrouped ECOR strains, as defined by multilocus enzyme electrophoresis (6). To provide a large, phylogenetically defined strain panel with which the new CGA PCR assay could be validated, six group D ECOR strains (6) and 132 other phylogenetically diverse *E. coli* isolates from humans, animals, and/or environmental sources (138 isolates total) were used. Included were CGA isolates and geographically matched non-CGA isolates from two previously described multicenter studies (11, 16) and from diverse other domestic and international locales (Buffalo, N.Y.; Cleveland, Ohio; Seattle, Wash.; Chicago, Ill.; Tucson, Ariz.; Billings, Mont.; Houston, Tex.; Birmingham, Ala.; Baltimore, Md.; Iowa City, Iowa; and Brazil, England, France, Germany, Israel, Spain, Sweden, and Thailand). The CGA status of these isolates was defined by RAPD analysis and, independently, by ERIC2 PCR, as described below.

RAPD analysis and ERIC2 PCR. RAPD analysis and ERIC2 PCR were performed as described previously (11, 16). For RAPD analysis (done in the laboratory of James R. Johnson, Minneapolis, Minn.), isolates were defined as representing CGA if their profiles were indistinguishable from those of known CGA controls according to the results of RAPD analysis with both of two RAPD primers or three or more of the five RAPD primers selected from among decamers 1247, 1254, 1281, 1283, and 1290 (1). For the ERIC2 PCR (done in the laboratory of Lee W. Riley, Berkeley, Calif.), isolates were defined as represent-

ing CGA if they yielded the previously described four-band CGA profile, in the absence of other prominent bands, irrespective of the presence of faint variable bands (16). Profiles were assessed by visual inspection of images of ethidium bromide-stained agarose gels containing molecular size standards, with or without computer assistance. All determinations were done in duplicate and included appropriate positive and negative controls.

Sequence analysis. The partial coding sequences (442 to 536 bp per gene) of eight *E. coli* housekeeping genes, i.e., *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *metG*, *purA*, and *recA*, were determined bidirectionally for the 4 CGA reference isolates and 28 other phylogenetically diverse *E. coli* isolates by using internal gene-specific primers, as communicated by Thierry Wirth (Max-Planck Institut, Berlin, Germany). The sequences were individually aligned for each gene by using the CLUSTAL-X program. Single-gene dendrograms were inferred according to the maximum-parsimony method by using the PAUP* program. Aligned sequences were inspected to identify putative CGA-specific single-nucleotide polymorphisms (SNPs).

Gene-specific PCR. Boiled lysates were used as the template DNA (13). Amplification was done in 0.6-ml tubes with 25-μl reaction mixtures containing 0.6 μM each primer, 0.8 mM deoxynucleoside triphosphates, 4 mM $MgCl_2$, 1× commercial buffer, 1.25 U of thermally activated *Taq* polymerase, and 2 μl of boiled lysate. The cycling protocol was 95°C for 10 min; then 25 cycles of 94°C for 30 s, 63°C for 30 s, and 68°C for 3 min; and then 72°C for 10 min. The 63°C annealing temperature was used for compatibility with our established multiplex virulence genotyping PCR assay (13). To assess the stability of the assay, PCR was done over a range of conditions, i.e., with various concentrations of each amplification mixture ingredient and at annealing temperatures from 59 through 67°C.

RESULTS

Identification of putative CGA-specific SNPs. In each of the eight dendrograms generated on the basis of the individual housekeeping genes, the four reference CGA strains were consistently placed together within a monophyletic clade. This clade variously included ECOR strain 47 in dendrograms generated with seven genes (e.g., *fumC*) (Fig. 1) and ECOR strain 44 and/or representatives of the O15:K52:H1 clonal group in dendrograms generated with five genes (data not shown). Inspection of the corresponding single-gene sequence alignments identified multiple SNPs in several genes that differentiated the CGA reference isolates from certain of the comparison strains. However, most of these SNPs were shared with multiple other isolates, in various combinations. Only *fumC* exhibited SNPs that corresponded closely with those of the CGA reference isolates, and even these were shared with ECOR strain 47 (Table 1). Consequently, ECOR strain 47 was assessed for CGA status by PCR fingerprinting. By ERIC2 PCR, ECOR strain 47 was indistinguishable from the CGA reference strains (Fig. 2), whereas according to RAPD analysis with five different RAPD primers, it was either highly similar to (four primers) or indistinguishable from (primer 1281) the CGA reference strains (Fig. 3). This confirmed that ECOR strain 47 is either a member of CGA (by ERIC2 PCR) or quite closely related to CGA (by RAPD analysis). Therefore, the three candidate *fumC* SNPs were provisionally regarded as CGA and ECOR strain 47 specific and were explored as targets for the CGA-specific PCR.

Selection and validation of CGA-specific primers. A candidate CGA-specific *fumC* forward primer with the contiguous SNPs G270A and C271T at its 3′ terminus proved to be largely specific to CGA (and *E. coli* reference strain 47) when it was used in PCR together with a consensus reverse *fumC* primer, primer CGAr (5′-CGTGCATCGCCGTTGGAAAG-3′). However, this primer pair reacted with several other (non-CGA) group D-derived ECOR strains, including ECOR strains 35 and 46 (data not shown). Upon sequence analysis, ECOR strains 35 and 46 were also found to exhibit the two *fumC* SNPs described above (Table 1). Therefore, an alternative candidate CGA-specific primer, i.e., primer CGAf (5′-GCTATCTGGCAGACT-3′), that had the third putative CGA-specific *fumC* SNP, i.e., C288T, at its 3′ terminus was devised. This second-generation CGA primer, in combination with reverse primer CGAr, yielded the predicted 175-bp band with the four CGA reference isolates and ECOR strain 47, yet it failed to react with any of several non-CGA control strains, including the (non-CGA) group D ECOR strains that did react with the initial putative CGA-specific primer. Accordingly, this primer pair was next tested with the entire validation set.

Among the 138 validation set isolates, the results of RAPD analysis and ERIC2 PCR concurred precisely as to CGA status, identifying 63 isolates as CGA and 75 isolates (including the 5 non-ECOR strain 47 group D ECOR strains) as non-CGA (Fig. 4). With the amplification of a 175-bp product

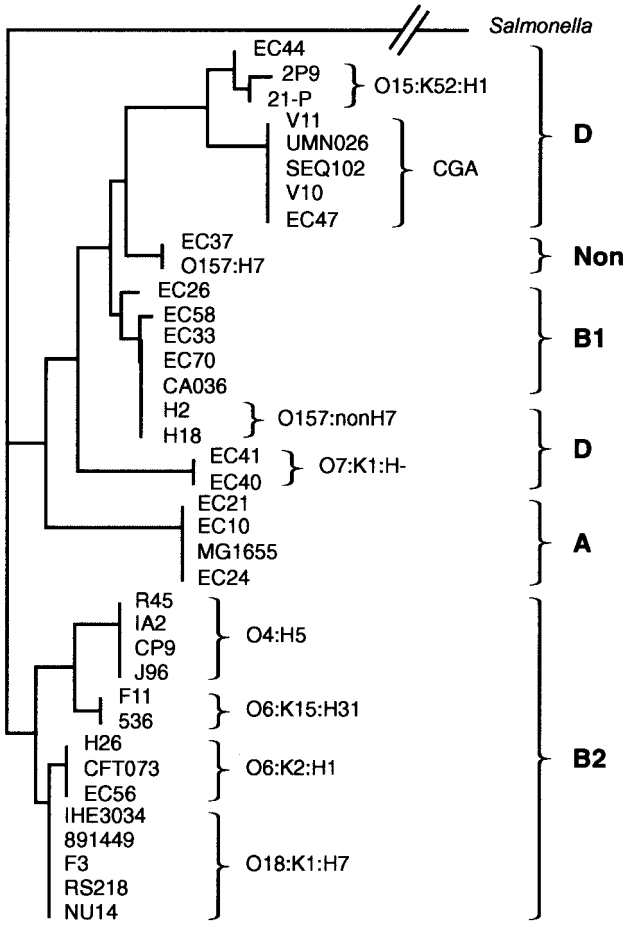

FIG. 1. Phylogenetic relationships among diverse *E. coli* isolates as inferred from the *fumC* sequence. The tree was inferred from the partial coding sequence for *fumC* by the neighbor-joining method. Major phylogenetic groups (groups A, B1, B2, and D and nonaligned strains [Non]) are labeled in bold at the far right and bracketed; O:K:H serotypes and CGA are bracketed and labeled immediately adjacent to the tree. *Salmonella* was used as an outgroup to root the tree. EC, ECOR strain.

TABLE 1. Distribution of *fumC* sequence polymorphisms among 36 *E. coli* isolates

Index strain for *fumC* sequence type (ECOR group of origin)[b]	No. of independent isolates with same *fumC* sequence type	Nucleotide at the following candidate CGA-specific positions within *fumC*[a]:		
		270	271	288
MG1655 (group A)	4	G	C	C
CFT073 (group B2)	8	G	T	C
CP9 (group B2)	4	G	C	C
536 (group B2)	2	G	C	C
33 (group B1)	5	G	C	C
58 (group B1)	1	G	C	C
2P9 (group D)	2	G	C	C
44 (group D)	1	G	C	C
UMN026/CGA (group D)[c]	**4**	**A**	**T**	**T**
47 (group D)	1	A	T	T
35 (group D)[d]	1	A	T	C
46 (group D)[d]	1	A	T	C
37 (ungrouped)	2	G	C	C

[a] All three polymorphisms are synonymous; i.e., they do not alter the peptide sequence.

[b] *fumC* sequence groups were defined by the presence of one or more SNPs within *fumC* in comparison with the sequences of the other isolates. The ECOR group of origin was defined by multilocus enzyme electrophoresis ECOR strains 33, 58, 44, 47, 35, 46, and 37) (8) or multiplex PCR (the other isolates) (4).

[c] Data for CGA reference isolates are shown in boldface. These isolates included UMN026, SEQ102, V10, and V11 (13, 16).

[d] ECOR strains 35 and 46 were sequenced only after they were discovered to react with the initial putative CGA-specific primers.

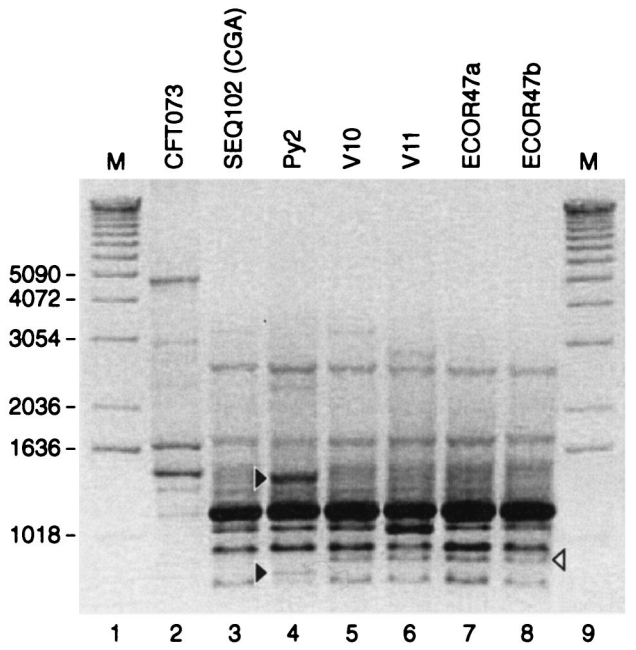

FIG. 2. ERIC2 PCR profiles of diverse *E. coli* strains, including *E. coli* reference strain (ECOR) 47. SEQ102 (lane 3) is a CGA reference isolate. Urosepsis isolates V10 and V11 (lanes 5 and 6, respectively) and ECOR strain 47 (versions a and b; lanes 7 and 8, respectively) exhibit profiles indistinguishable from that of SEQ102, except for one irreproducible band (white arrowhead). In contrast, strain Py2 exhibits two reproducible bands (black arrowheads) not present in SEQ102. Non-CGA strain CFT073 (lane 2) is shown for reference.

scored as a positive result and the absence of amplification scored as a negative result, the new CGA-specific primers yielded unambiguous results that concurred precisely with the ERIC2 PCR fingerprinting-based CGA assessments, thus providing an estimated sensitivity and specificity of 100% each (Table 2).

Interlaboratory reproducibility and stability of assay. The assay was successfully established in two laboratories other than the authors' on the basis of a protocol communicated by the authors, in one instance with the newly synthesized primers and in the other with primers provided by the authors (E. W. Rice and E. N. Janoff, personal communications). In one of these laboratories (that of E. N. Janoff), blind testing of a panel of 10 CGA and 10 non-CGA reference isolates (provided by the authors) yielded results which corresponded precisely with those obtained in the laboratory of J. R. Johnson and, hence, with the results of the ERIC2 PCR and RAPD fingerprinting (data not shown).

Different PCR conditions, including halving and doubling of the concentrations of all components of the reaction mixture (individually) and the use of annealing temperatures ranging from 59 to 65°C, had negligible effects on amplification of the desired 175-bp CGA-specific product (data not shown). No amplification was observed when the annealing temperature was raised to 67°C.

DISCUSSION

The new CGA-specific primers, which were selected on the basis of a newly identified SNP within *fumC* (C288T), detected CGA members with extreme precision within a large and phylogenetically diverse set of *E. coli* isolates among which CGA status was defined by established molecular methods, i.e., RAPD analysis and ERIC2 PCR fingerprinting. The new assay was easy to use, was tolerant of various PCR conditions, and yielded concordant results when the assays were assessed in a blinded fashion in two different laboratories. The assay should thus make detection of CGA readily available to any laboratory with a diagnostic PCR capability, without the well-recognized pitfalls of PCR-based fingerprinting methods, such as intrinsic assay variability and subjective interpretation of complex banding patterns (3, 5, 12, 19, 21, 22).

Reliance on a single SNP to identify a particular clonal group would seem potentially hazardous, given the theoretical possibility that this SNP could appear in a phylogenetically unrelated clone or be absent from a member of CGA due to a point mutation or horizontal transfer (7). However, neither of these phenomena was observed with our validation set, for which (except for the special case of ECOR strain 47) the results of the new assay exhibited 100% concordance with those of two whole-genome typing methods, i.e., ERIC2 PCR and RAPD analysis. Thus, it would seem that despite its theoretical vulnerability, the new assay is sufficiently robust for standard molecular epidemiological applications. If a point mutation or recombinational event were to occur at the critical position in *fumC*, thereby rendering a non-CGA member falsely positive by our assay or a CGA member falsely negative, so long as this change did not confer a fitness advantage (as it should not, with a housekeeping gene), this should remain an

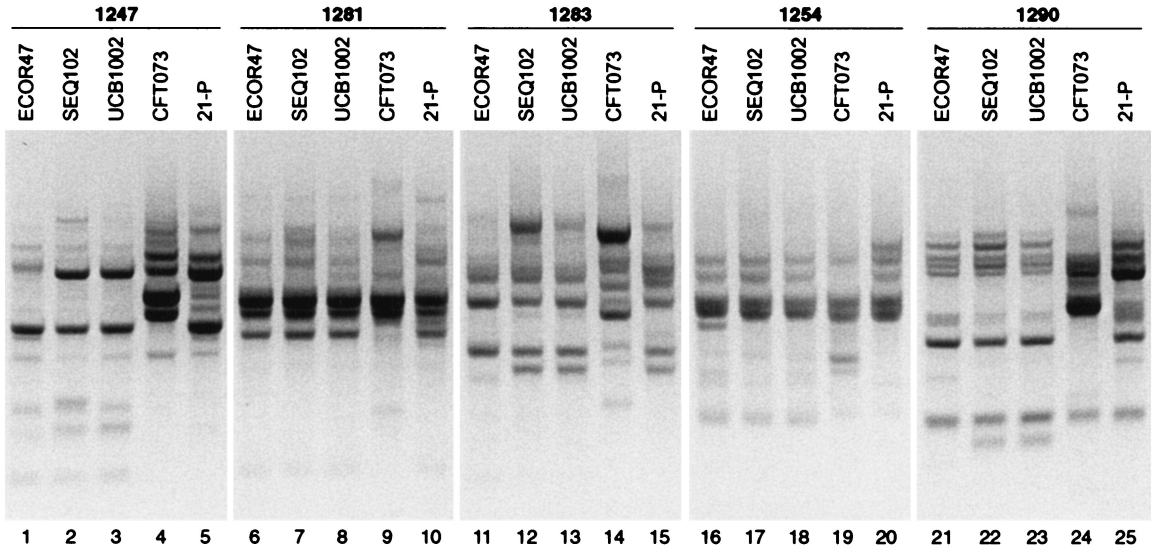

FIG. 3. RAPD profiles of diverse *E. coli* strains, including *E. coli* reference strain (ECOR) 47. The profiles were generated with arbitrary decamer primers 1247, 1281, 1287, 1254, and 1290, as listed above the gel lanes. ECOR strain 47 (lanes 1, 6, 11, 16, and 21) closely resembles, but (except with primer 1281) is distinct from, CGA reference isolates SEQ102 and UCB1002 (lanes 2, 7, 12, 17, and 22 and lanes 3, 8, 13, 18, and 28, respectively). Strain 21-P (O15:K52:H1; lanes 5, 10, 15, 20, and 25) is similar to the CGA reference isolates, especially with primers 1281, 1287, and 1254. Non-CGA strain CFT073 (lanes 4, 9, 14, 19, and 24) is shown for reference.

isolated event that would not appreciably affect the assay's overall sensitivity or specificity.

The minor discrepancy noted between ERIC2 PCR fingerprinting and RAPD analysis with respect to the CGA status of ECOR strain 47 was not otherwise encountered among the 138 test strains. Thus, although this phenomenon does suggest a potential problem regarding the precise definitional boundaries of CGA and the appropriate molecular methods for its detection, this seems unlikely to present a practical dilemma during the analysis of wild-type clinical or environmental isolates, among which such discrepancies appear to be rare to nonexistent. Accordingly, all three methods used here, i.e., ERIC2 PCR, RAPD analysis, and CGA-specific PCR, appear to be similarly able to identify members of CGA in a real-world context. The choice of which assay to use would depend on the preferences of the individual laboratory.

Interestingly, ECOR strain 47 is a pre-1973 sheep isolate from New Guinea that is broadly antimicrobial susceptible, lacks most extraintestinal virulence factors, and reportedly exhibits serotype O-multiple:H18 (9, 17, 20). Our serendipitous discovery that ECOR strain 47 is a close relative (possibly a recent ancestor) or early member of CGA has implications for understanding CGA's evolutionary origins. It can be speculated that this particular genomic background had evolved at least by the early 1970s but lacked the virulence traits and resistance markers that characterize today's clinical CGA isolates, which, of note, usually exhibit serotypes O11:H18, O17/77:H18, or O73:H18 (11, 16). At some point during the subsequent two decades a member of this clonal group acquired

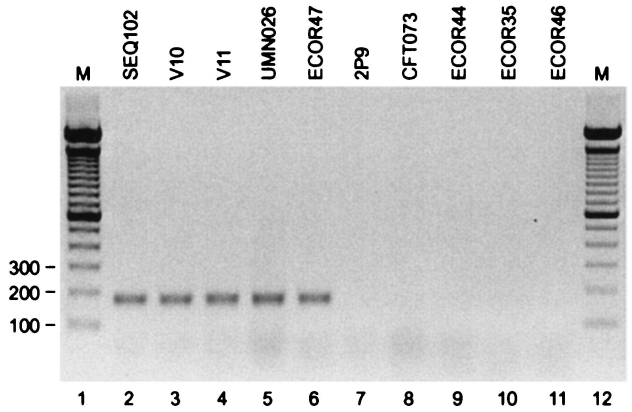

FIG. 4. Gene-specific PCR for representative of *E. coli* CGA isolates, *E. coli* reference (ECOR) strain 47, and selected non-CGA reference isolates. The confirmed CGA members (lanes 2 to 5) and ECOR strain 47 (lane 7) all yielded the predicted 175-bp band, whereas non-CGA reference strains (lanes 8 to 12) did not. Lane M, marker (100-bp ladder). Sizes (in base pairs) are shown on the left.

TABLE 2. Concordance of results of gene-specific PCR with those of two established genomic fingerprinting methods in identifying CGA among 138 *E. coli* isolates

CGA status by:		No. of isolates with pattern	No. of isolates with the following CGA status by gene-specific PCR[a]	
RAPD PCR	ERIC2 PCR		CGA (n = 63)	Non-CGA (n = 75)
+	+	62	62	0
−	−	75	0	75
−[b]	+	1[b]	1	0

[a] Gene-specific PCR was done with forward primer CGAf, which targets the C288T *fumC* polymorphism, and consensus reverse primer CGAr.

[b] With four of five RAPD primers, ECOR strain 47 exhibited RAPD profiles highly similar to, but distinct from, those of the CGA reference isolates.

via horizontal transfer, perhaps from an *E. coli* O15:K52:H1 donor (11), the relevant chromosomal virulence factors and plasmid-associated antimicrobial resistance genes. This was followed by expansion and dissemination of the now more fit clonal variant, which in turn gave rise to the widespread occurrence of drug-resistant urinary tract infections in the late 1990s that recently brought CGA to attention (11, 16). Further analysis of archival collections, which should be facilitated by the new CGA-specific assay, should help refine this hypothesized scenario for the emergence of CGA.

In summary, we have devised and validated a CGA-specific PCR assay, based on a single SNP within *fumC* (C288T), that allows the rapid and specific detection of the recently described *E. coli* CGA. This assay should facilitate the studies needed to define the prevalence, distribution, ecology, and epidemiology of CGA and to trace the emergence of CGA as disseminated multidrug-resistant extraintestinal pathogens.

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